Reprinted from the Proceedings of the NATIONAL ACADEMY OF SCIENCES
Vol. 47, No. 1, pp. 52–55. January, 1961.

*LINKAGE OF GENETIC UNITS OF BACILLUS SUBTILIS IN DNA TRANSFORMATION**

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Communicated November 25, 1960

Bacterial transformation, the transfer of genetic markers by DNA, provides a mechanism by which closely linked genetic loci may be ordered in a segmental map. Since a unit of DNA transferred from a donor bacterium to the recipient cell represents only a small fragment of the entire genome,[1] it is not possible to arrange many randomly distributed loci in any single transfer experiment. By the same token, when a pair of genes shows an appreciable degree of joint transformation, it points directly to their genetic contiguity. During a genetic and chemical study of the system of DNA transfer in *Bacillus subtilis* described by Spizizen,[2] it was observed that two markers could be simultaneously incorporated into a doubly auxotrophic recipient. DNA isolated from a wild type organism will transfer two independent characters—one concerned with an enzyme of tryptophan (more precisely indole glycerol phosphate) formation and the other with an enzyme of histidine biosynthesis. The evidence for their linked transfer is presented below.

Materials and Methods.—The origin and genotype of the principal strains employed in this study are given in Table 1. The liquid media employed include: Pen, Penassay Broth currently

TABLE 1

LIST OF STRAINS OF *Bacillus subtilis*

Strain	Genotype	Origin
168	*ind*$^-$	Burkholder and Giles[3]
23	*thr*$^-$	Burkholder and Giles
SB19	Reference prototroph	23 — × 168*
SB1	*ind*$^-$ his$_1^-$	UV treatment[4] of strain 168
SB25	*ind*$^-$ *his*$_2^-$	UV treatment[4] of strain 168
SB48	*his*$_3^-$	SB19 — × 168 (nitrous acid treatment)[5]
SB60	*ind*$^-$ *his*$_3^-$	168 — × SB48[5]
SB32	*his*$_2^-$	SB19 — × SB25
SB33	*ind*$^-$	SB19 — × SB25
SB31	Prototroph	SB19 — × SB25

* The symbol 23 — × 168 indicates that DNA from strain 23 was used to transform the recipient strain 168. SB31, SB32, and SB33 are transformants selected to insure an isogenic background for the *ind* and *his* markers.

sold as Difco Antibiotic Medium 3; CHT-2, minimal medium[2] containing 0.02 per cent acid hydrolyzed casein and 20 μg per ml of DL-tryptophan; CHT-10, minimal medium containing 0.1 per cent casein hydrolysate and 200 μg per ml of DL-tryptophan. For platings, we used minimal agar[6] (histidine agar or tryptophan agar) containing 40 μg per ml L-histidine or 20 μg per ml DL-tryptophan, respectively.

The recipient cells are grown aerobically[7] to the completion of exponential growth in Pen medium. The culture is washed and resuspended, 1:10 in a 15 × 125 mm test tube containing 5 ml of CHT-2 medium. After four hours of further incubation at 37°, the cells are washed and diluted 1:10 into fresh CHT-10 medium precooled to 30°, and 0.9 ml aliquots are dispensed into 15 × 125 mm test tubes. After incubation at 30° for 90 minutes, 0.1 ml of a solution of DNA (containing in most instances 1 μg per ml of DNA) is added to each tube, and incubation is continued for an additional 30 minutes. Deoxyribonuclease (Worthington Biochemical Corp.)

20 μg, and magnesium sulfate to give a concentration of 0.01 M are added. After an additional 10 minutes incubation, 0.1 ml aliquots of the cells are plated on the indicated minimal agar. These plates are incubated at 37°, and read at 40 to 48 hours. This procedure, a modification of the technique devised by Spizizen,[8] has given consistent results in our hands.

For the preparation of DNA, the donor cells are grown in Pen and washed twice in 0.14 M NaCl, and the packed cells are resuspended at a concentration of 1 gm (wet weight) per 4 ml of a solution containing 0.14 M sodium chloride, 0.01 M sodium citrate, and 0.01 M phosphate buffer solution, pH 6.6. Lysozyme (Armour) is added to give a concentration of 1 mg per ml and the suspension stirred at 37° for 10 minutes. The now viscous lysate is suspended in 10 volumes of a solution of 0.14 M sodium chloride and 0.01 M sodium citrate at 0°. The nucleic acid-protein complex is then sedimented at 0° at 30,000 $\times$ g for 20 minutes. The pellet is suspended in 4 volumes of 2 M NaCl relative to the original volume of the lysate and extracted by agitation with a magnetic stirrer for 30 minutes at 4°. The suspension is then centrifuged at 20,000 $\times$ g for 20 minutes at 0° and the pellet reextracted with 2 M NaCl. The pooled extracts are added to 2 volumes of 95 per cent ethanol previously cooled to -20°. The fibrous precipitate of DNA is collected on a hooked rod and resuspended in 2 M NaCl. After storage overnight at 4°, the DNA is deproteinized by gentle manual shaking with an equal volume of a chloroform-octanol (5:1) mixture for 10 minutes, followed by centrifugation at 2,500 $\times$ g for 5 minutes. The upper, aqueous layer is transferred to fresh chloroform-octanol and the process repeated (usually seven times) until no gelatinous interface between the two layers appears. The DNA is reprecipitated in 2 volumes of 95 per cent ethanol and dissolved in 2 M NaCl + 0.01 M phosphate buffer, pH 7.4. In this medium, the DNA has been stored for at least 6 months at 4° and has retained full transforming activity. However, repeated pipetting of the DNA solution often results in a loss in activity, perhaps due to shear forces.[9]

The procedure as described here is a modification of the procedures employed by Spizizen[8] and Ephrati-Elizur.[10] When prepared by this method, 0.02 to 0.05 μg of DNA will saturate 7×10^7 recipient cells in one ml.

Experimental Results.—Nonidentity of his mutants: The relationship of the single histidine markers to each other was studied by determining whether the DNA extracted from one *his*$^-$ mutant could transform another *his*$^-$ mutant to prototrophy (Table 2). If prototrophs do result, the mutant loci are recombinationally distinct, i.e., they do not mark the same point of the chromosome. The inference from Table 2 is that these three *his*$^-$ strains carry nonallelic mutations.

TABLE 2

DNA TRANSFERS INVOLVING *his* LOCI

Donor DNA from	Recipient Bacteria SB1	SB25	SB48
SB1 (*his*$_1^-$)	0	60	86
SB25 (*his*$_2^-$)	21	0	15
SB48 (*his*$_3^-$)	33	31	0
SB19 (*his*$^+$)	226	98	190

The numbers are *his*$^+$ transformants per μl aliquot of recipient cell suspension (5×10^7 per ml). They were scored on tryptophan agar. The quantitative significance of the numbers is uncertain on account of possible variations in the competence of the recipient cultures and the absolute activity of the donor DNA.

Linkage: Although a comparison of the absolute frequencies of transformation to prototrophy could serve to estimate the relative distances of the mutant loci, as in Table 2, there are two important reservations: (1) the need to compare trials involving different, perhaps not comparable, batches of cells and of DNA and (2) the untested assumption that many transformants are produced with the donor markers which cannot be counted in the scoring system. A more reliable method depends on the actual recovery and estimation of single and multiple marker transfers.

The linkage relationship can be expressed as the cotransfer index, r, a measure

of the frequency of joint transfer of two markers compared to the total number of recombinant genotypes measured by the transformation experiment. To use a general notation,[11] in a system a^1b^1—× $a^\circ b^\circ$, giving transformant types a^1b^1, a^1b°, and $a^\circ b^1$,

$$r = \frac{a^1b^1}{a^1b^1 + a^1b^\circ + a^\circ b^1} = \frac{a^1b^1}{a^1 + b^1 - a^1b^1} \quad (\text{since } a^1 = a^1b^\circ + a^1b^1).$$

For example, in Table 3,

$$r = \frac{ind^+ his^+}{ind^+ + his^+ - ind^+ his^+}.$$

In other experiments, it may be possible to estimate a^1b^1 and a^1b° but not $a^\circ b^1$. In this case, we may assume $a^\circ b^1 \cong a^1b^\circ$ and write an estimated index

$$r_a = \frac{a^1b^1}{a^1b^1 + 2\,a^1b^\circ}.$$

For unlinked markers, r can be estimated as $a^1b^\circ/2N$, i.e., one-half the efficiency of transformation for either marker, N being the total number of (transformable) cells.

The ability of DNA extracted from wild type cells to accomplish the joint transformation of the three strains of *ind*$^-$*his*$^-$ cells to prototrophy is shown in Table 3.

TABLE 3

TRANSFORMATION OF *ind*$^-$ *his*$^-$ AUXOTROPHS TO PROTOTROPHY

Donor DNA		Recipient Cells	Transformant Classes, per 10^4 recipient cells: *ind*$^+$	*his*$^+$	*ind*$^+$ *his*$^+$ Found	*ind*$^+$ *his*$^+$ Calculated as random coincidence	Cotransfer Index r
SB31 *ind*$^+$ *his*$^+$	— ×	SB25 *ind*$^-$ *his*$_2^-$	70	72	48	0.5	0.51
SB31 *ind*$^+$ *his*$^+$	— ×	SB60 *ind*$^-$ *his*$_3^-$	3.9	5.0	0.03	0.02	0.003
SB31 *ind*$^+$ *his*$^+$	— ×	SB1 *ind*$^-$ *his*$_1^-$	50	100	0.8	0.5	0.005
SB32 *ind*$^+$ *his*$_2^-$ + SB33 *ind*$^-$ *his*$^+$	—×	SB25 *ind*$^-$ *his*$_2^-$	46	20	0.2	0.09	0.003

The transformation procedure was as given in the protocols except that DNA was used at a concentration of 20 μg per ml for *his*$_1$ and *his*$_2$ and at a concentration of 0.1 μg per ml for *his*$_3$. The transformant classes were counted on minimal agar (for *ind*$^+$ *his*$^+$), histidine agar (for *ind*$^+$) and tryptophan agar (for *his*$^+$), respectively. The calculated random coincidence is the product of *ind*$^+$ × *his*$^+$.

The cotransfer index for the appearance of double transformants *ind*$^+$*his*$^+$ varies from 0.004 to 0.51 with different *his* mutants. This result is taken as evidence of linkage between the *ind* and *his*$_2$ loci. Introducing the *ind*$^+$ and *his*$^+$ markers on different DNA molecules by mixing DNA preparations from *ind*$^+$*his*$_2^-$ gave no evidence of linkage. This argues against the possibility that the linkage can be accounted for by the random coincidence of unlinked markers.

Additional auxotrophic markers have been introduced into strain 168 after UV irradiation or nitrous acid treatment of DNA *in vitro* or by selection of spontaneously occurring mutants as previously described. These markers included growth requirements for methionine, methionine plus lysine, glutamic acid or proline, valine plus isoleucine, cystine, and a streptomycin resistance marker. When limiting concentrations of SB19 DNA were used, the cotransfer index never exceeded 0.005. Thus, while *ind* is closely linked to *his*$_2$, it is not linked to any of six other markers.

Discussion.—The foregoing evidence indicates that the loci his_2 and *ind* stand in a special relationship to each other in contrast with several other pairs of loci studied in the same way.

The cotransfer index for these markers might be accounted for if the transformable cells were a very small fraction of the total. The estimation of competence by the addition of increasing amounts of DNA is not a satisfactory measure owing to the possible interference of one absorption with another. The main argument against this interpretation of the high cotransfer index for ind/his_2 is its unique value for these markers compared to other sets and to the introduction of ind^+ and his^+ from separate DNA preparations. The evidence is closely analogous to that for the linkage of the *Fla* and H_1 loci in Salmonella transduction[12] and for mannitol dehydrogenase and streptomycin resistance in Pneumococcus transformation.[1]

The present findings, in conjunction with those of Ephrati-Elizur *et al.*,[13] are the first evidence of linkage in *B. subtilis*. This type of analysis, hopefully, may open the way to more intensive studies such as have been done on the genetic chemistry of DNA in Pneumococcus on the one hand and on the sequential arrangement of genes and their physiological function in the enteric bacteria on the other. In fact, the histidine and tryptophan pathways have been studied especially extensively in Salmonella, where a striking correlation between linkage and biosynthetic sequence has been observed.[14] At first sight, such a correlation could not be inferred from the present data, but this question requires more complete biochemical and genetic study.

* This work was supported by training grant 2G295 and research grant C4496 from the National Institutes of Health and by a grant from the National Science Foundation.

† The work was done while the author was on a fellowship awarded by the American Cancer Society.

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